

Reduced arbuscular mycorrhizal root colonization in *Tropaeolum majus* and *Carica papaya* after jasmonic acid application can not be attributed to increased glucosinolate levels

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Summary

The plant signal compounds jasmonic acid or salicylic acid were applied as abiotic elicitors to leaves of glucosinolate-containing members of the Tropaeolaceae (*Tropaeolum majus*) and Caricaceae (*Carica papaya*) and to leaves of the glucosinolate-free plant cucumber. Both compounds are known to induce the accumulation of glucosinolates in *Brassica* plants. In roots of glucosinolate-containing plants the two compounds enhanced glucosinolate levels or new glucosinolates were accumulated. In all plants treated with jasmonic acid a reduction of root colonization by the arbuscular mycorrhizal fungus *Glomus mosseae* was observed. No such effect occurred after the salicylic acid treatment.

In addition, members of the glucosinolate-containing Tropaeolaceae family were inoculated with the arbuscular mycorrhizal fungus (AMF), and later on the glucosinolate content was determined in roots of mycorrhizal and in roots of non-mycorrhizal plants. Root colonization by the AMF resulted in a large increase of the glucosinolate content, however, the glucosinolate levels in mycorrhizal and in non-mycorrhizal plants showed no effect on root colonization by the AMF.

From our results we concluded that the glucosinolate levels can not generally be linked to the non-host status of glucosinolate-containing plants, however, a role of specific glucosinolates in the expression of the non-host status of glucosinolate-containing plants can not be excluded. Moreover, we found that the application of jasmonic acid highly suppresses mycorrhization and this suppression is not a glucosinolate-dependent mechanism.

Key words: arbuscular mycorrhiza – *Carica papaya* – cucumber – glomales – glucosinolate – jasmonic acid – salicylic acid – Tropaeolaceae

Abbreviations: AMF = arbuscular mycorrhizal fungus. – GSL = glucosinolate. – JA = jasmonic acid. – SA = salicylic acid

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Introduction

Arbuscular mycorrhizal fungi (AMF) colonize roots thereby improving plant nutrition primarily by increasing phosphate transport from the soil to the plant, while the host plant provides the fungi with carbohydrates. Most land plants are known to form the AM symbiosis, however, there are some exceptions (Smith and Read 1997). Glucosinolates (GSLs) are secondary plant compounds found in plants of the AM non-host families of the Brassicaceae and the Resedaceae and in plants of the AM host families of the Caricaceae and Tropeaeaceae (Harley and Harley 1987, Vierheilig et al. 2000). The enzyme myrosinase (thioglucosidase; EC 3.2.3.1), found in all tissues of GSL-containing species, hydrolyses a range of GSLs to compounds with fungitoxic or fungistatic activity (Bennett and Wallsgrove 1994, Fahey et al. 2001, Greenhalgh and Mitchell 1976, Vierheilig and Ocampo 1990a, Wallsgrove et al. 1999). Thus, the presence of certain GSLs in plants has been proposed to play a role in the resistance of GSL-containing plants against phytopathogenic fungal invaders (Doughty et al. 1991, Ludwig-Müller et al. 1997, 1999, Wallsgrove et al. 1999) and against the AM fungal root symbiont (El-Atrach et al. 1989, Schreiner and Koide 1993a, b, Vierheilig and Ocampo 1990a, b, Vierheilig et al. 1995, 1998a).

Recently, Vierheilig et al. (2000), studying a wide range of GSL-containing plants, suggested that a specific GSL-pattern might be responsible for the AM non-host status of most GSL-containing plants. Thus it was tempting to speculate that changes of the GSL pattern of GSL-containing host plants could alter the host status of these plants to AMF.

Quantitative and qualitative changes of the GSL pattern have been reported in leaves (Doughty et al. 1995, Kiddle et al. 1994, Ludwig-Müller et al. 1997) and roots (Ludwig-Müller et al. 1997) of GSL-containing plants after the application of jasmonic (JA) or salicylic acid (SA) and in roots of GSL-containing plants after inoculation with AMF (Vierheilig et al. 2000).

In the present study we investigated whether altered GSL patterns in roots can be linked to the plant's ability/inability to form the AMF symbiosis.

Materials and Methods

Biological material and growing conditions

Source of plant material: *Tropaeolum majus* L. Bot. Garden was obtained from the Botanical Garden, Frankfurt, Germany, all other *Tropaeolum* seeds were from Kings (E. W. King & Co. Ltd.), Monks Farm, Kelvedon, Essex, UK. Seeds of *Carica papaya* L. were from local markets.

Seeds of GSL-containing plants *T. majus*, *T. canariense* L., *Carica papaya* L. and a GSL-free control plant, cucumber (*Cucumis sativus* L. cv. Straight Eight), were surface-sterilized by soaking in 0.75% sodium hypochlorite for 5 min, rinsed with tap water and germinated in vermiculite.

After germination 10 d-old seedlings were transferred to a steam-sterilized (40 min, 120 °C) mixture of silicate sand, TurFace (baked clay substrate which is mechanically broken into particles with a diameter of 2–5 mm; Applied Industrial Materials, Corp., Buffalo Grove, Illinois, USA) and soil (v:v:v/2:2:1) into the compartment system developed by Wyss et al. (1991). Plants were grown in the compartment system for 1 week before inoculation. Plants were inoculated by joining the lateral compartments containing the test plants with inoculum compartments (for details see Wyss et al. 1991) containing the AMF *Glomus mosseae* (Nicolson & Gerdemann) Gerd. & Trappe (BEG 12, La Banque Européenne des Glomales, International Institute of Biotechnology, Kent, GB). Inoculated and non-inoculated plants were grown in a growth chamber (day/night cycle: 16 h; 23 °C/8 h; 19 °C; rel. humidity 50%).

Every second day, after inoculation with the AMF, an aqueous solution of SA or JA was sprayed onto the leaves. Due to the waxy surface of *T. majus* leaves, the leaves had to be rubbed gently before spraying to assure a moistening of the leaf surface. Leaves of *T. majus* control plants were treated similarly. To avoid excess solution getting into the soil, the soil was covered with cotton wool before each spraying. *T. majus* plants were harvested 14 days after inoculation and *C. papaya* and cucumber plants 10 days after inoculation. Root and shoot fresh weight was determined at the end of the experiment.

Measuring root colonization

Several fresh roots from each plant were cleared by boiling in 10% KOH and cucumber roots were stained according to the method of Vierheilig et al. (1998b) by boiling in a 5% ink (Shaeffer, black)/5% v/v acetic acid solution, whereas roots of all other plants were stained by boiling in 0.05% trypan blue/vinegar 5% v/v acetic acid solution (Vierheilig and Piché 1998). Stained roots were observed with a light microscope and the percentage of root colonization was determined according to a modified method of Newman (1966).

Glucosinolate analysis

Plants were harvested, the roots washed with tap water, dried between filter paper and the fresh weight of each sample was recorded. The plant material was frozen in liquid N₂ before freeze-drying. Dry samples were milled to a fine powder prior to extraction for glucosinolate analyses. GSL extractions (from 3 × 40 mg dry weight sample⁻¹) and determinations were performed as previously described (Kiddle et al. 2001) using sinigrin as an extraction standard. Separation and detection of desulpho-GSLs was done using a Phenomenex Luna C₁₈ (2) (4.6 × 250 mm, 5 μm) column in combination with a Hewlett Packard HP1100 photo-diode array HPLC system and identification was achieved using authentic standards, which were previously identified by NMR (¹³C and ¹H) and chemical ionisation MS (Kiddle et al. 2001). All analyses were carried out in triplicate for each sample. Means and standard errors of means are given.

Results

The application of SA or JA (5 mmol/L) to leaves resulted in a strong accumulation of benzyl GSL in roots of *T. majus* and *C. papaya* (Table 1). The induction pattern for benzyl GSL was

Table 1. Effect of foliar-application of SA and JA (5 mmol/L) on the GSL content in roots of *T. majus* and *C. papaya* plants.

Species	Treatment	GSL	nmol g ⁻¹ DW
<i>T. majus</i> *	Control	benzyl	27441±307
		3-indolylmethyl	0
	+ SA	benzyl	57628±4238
		3-indolylmethyl	399±116
	+ JA	benzyl	57893±2091
		3-indolylmethyl	6011±787
<i>C. papaya</i>	Control	benzyl	11034±569
		3-indolylmethyl	trace
	+ SA	benzyl	26918±1899
		3-indolylmethyl	245±17
	+ JA	benzyl	31775±2077
		3-indolylmethyl	594±26

* cv. Botanical Garden

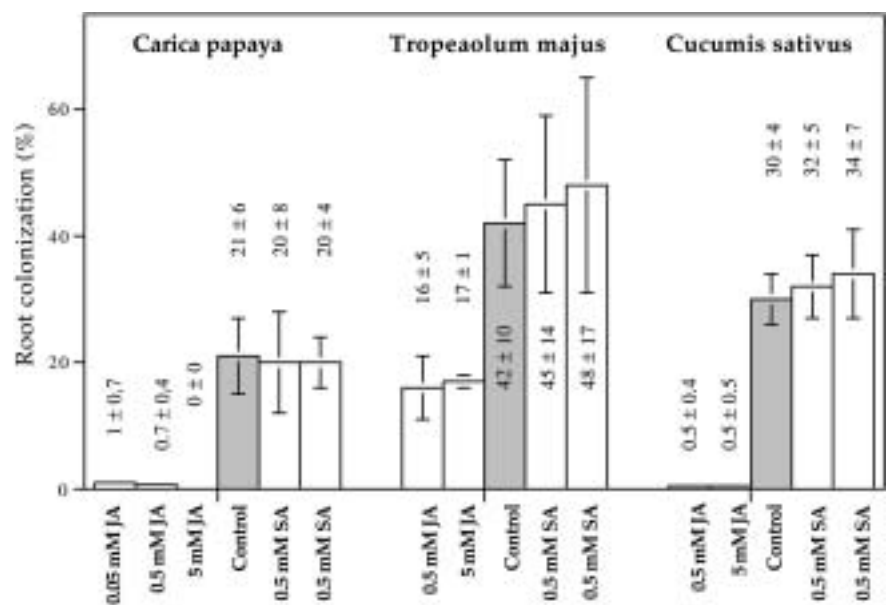
similar with SA and JA, but different for 3-indolylmethyl GSL. 3-indolylmethyl GSL was not detected in roots of untreated *T. majus*, but slightly accumulated in roots after SA foliar-treatment (399 ± 166 nmol g⁻¹, DW) and highly accumulated in roots after JA foliar-treatment (6011 ± 787 nmol g⁻¹, DW). In roots of untreated *C. papaya* only traces of 3-indolylmethyl GSL were found, whereas higher levels were observed in roots after SA (245 ± 17 nmol g⁻¹, DW) and JA (594 ± 26 nmol g⁻¹, DW) treatments. Naturally, no GSLs were detected in cucumber roots after the SA or JA treatments.

Plants treated with SA (0.5 mmol/L and 5 mmol/L) exhibited similar AMF colonization levels and characteristics as control plants, whereas all JA treatments (0.05 mmol/L, 0.5 mmol/L or

5 mmol/L) greatly reduced AMF root colonization in *T. majus*, *C. papaya* and cucumber (Fig. 1).

After application of 5 mmol/L SA to leaves, on all plants some leaf necroses occurred, whereas after the 5 mmol/L JA application only leaves of *C. papaya* turned slightly yellow. Leaf application of lower compound concentrations showed no effect. In *C. papaya* the 5 mmol/L JA application resulted in a drastic and the 0.5 mmol/L JA application in a slight reduction of the root fresh weight (Fig. 2). No significant effect on the root fresh weight could be observed in the other two plant species (Figs. 3 and 4) or in *C. papaya* with the lowest (0.05 mmol/L) JA concentration (Fig. 2). In cucumber and *C. papaya* the 5 mmol/L JA application reduced the shoot fresh weight. Foliar application of SA (5 mmol/L) significantly reduced the root and the shoot fresh weight in *T. majus* (Fig. 3) and the shoot fresh weight in cucumber (Fig. 4). In none of the tested plants, the lower SA concentration (0.5 mmol/L) had any effect, neither on the root nor on the shoot fresh weight (Figs. 2, 3 and 4). It can therefore be concluded that alterations in the overall performance of the plants, is not a factor responsible for AM suppression.

All plant species or varieties of the Tropaeolaceae were highly colonized by *G. mosseae* (Table 2). There was no clear correlation between the degree of root colonization and the GSL content in roots of non-inoculated plants, however, roots of the *T. majus* cultivar «Botanical Garden» with the highest content of benzyl GSL showed the lowest root colonization. In all mycorrhizal *Tropaeolum* plants a change in the GSL accumulation pattern compared to controls was observed. The content of benzyl GSL was 2 to 4-fold increased, whereas the content of 3-indolylmethyl GSL was either increased (in the cultivars Golden Gleam and Scarlet Gleam), decreased (in the cultivars Empress of India Dwarf variety, Empress of India

**Figure 1.** Percentage of root colonization by *Glomus mosseae* in *Carica papaya*, *Tropeaolum majus* and *Cucumis sativus* (cucumber) after foliar application of different concentrations of jasmonic or salicylic acid.

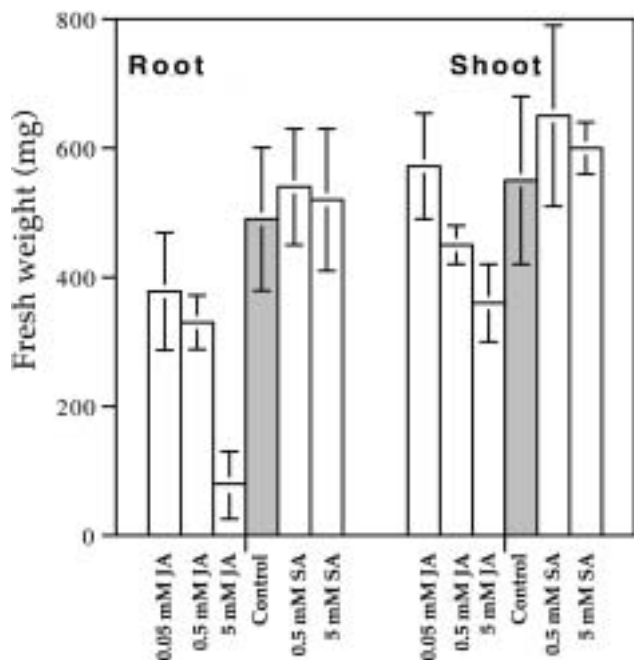


Figure 2. Root and shoot fresh weight of *Carica papaya* after foliar application of different concentrations of jasmonic or salicylic acid.

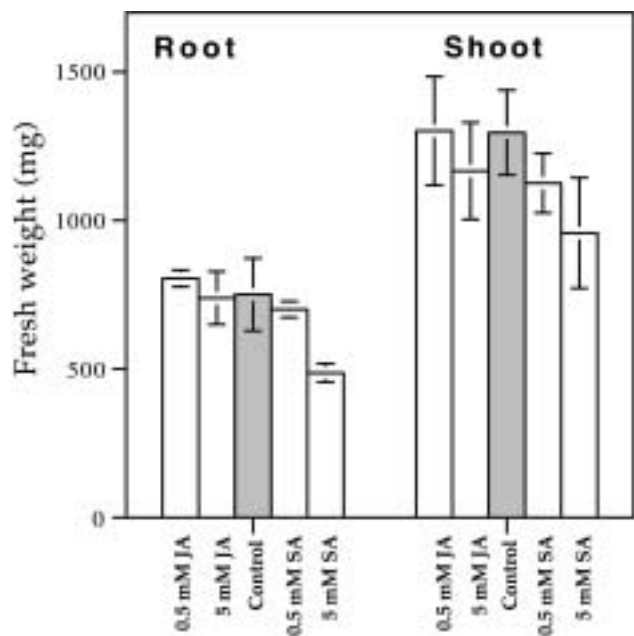


Figure 3. Root and shoot fresh weight of *Tropeaolum majus* after foliar application of different concentrations of jasmonic or salicylic acid.

Standard variety and Alaska) or was a *de novo* GSL (in the cultivar Botanical Garden).

Neither in mycorrhizal nor in non-mycorrhizal roots of *T. canariense* 3-indolylmethyl GSL was detected (Table 2). However, several other GSLs were found in significant amounts. In

mycorrhizal roots the content of aliphatic GSL decreased whereas concentrations of p-hydroxybenzyl GSL (9 fold) and p-methoxybenzyl GSL (more than 5 fold) were highly increased.

Discussion

Although GSLs themselves do not show any effect on fungi, it is known that hydrolysis products of nearly all GSLs exhibit a fungitoxic or fungistatic activity (Bennett and Wallsgrove 1994, Fahey et al. 2001, Greenhalgh and Mitchell 1976, Manici et al. 1997, Mithen and Lewis 1986, Vierheilig and Ocampo 1990a, Wallsgrove et al. 1999). It is tempting to speculate that changes in the glucosinolate pattern do translate in different hydrolysis products, thus, affecting the mycorrhizal status of a plant.

In our study with GSL-containing AM host plants, the application of the signalling compounds jasmonic and salicylic acid or the inoculation with an AMF resulted in alterations of the GSL pattern. In the first experiment, in roots of *T. majus* and *C. papaya*, foliar-treated with JA or SA, the GSL accumulation pattern was nearly similar. In treated plants not only the aromatic benzyl GSL was highly increased, but also the indole 3-indolylmethyl GSL was newly accumulated, demonstrating for the first time that the two signalling compounds can alter GSL patterns in other plant families apart from the Brassicaceae. Interestingly, although the GSL accumulation pattern was nearly similar with both compounds, only in JA

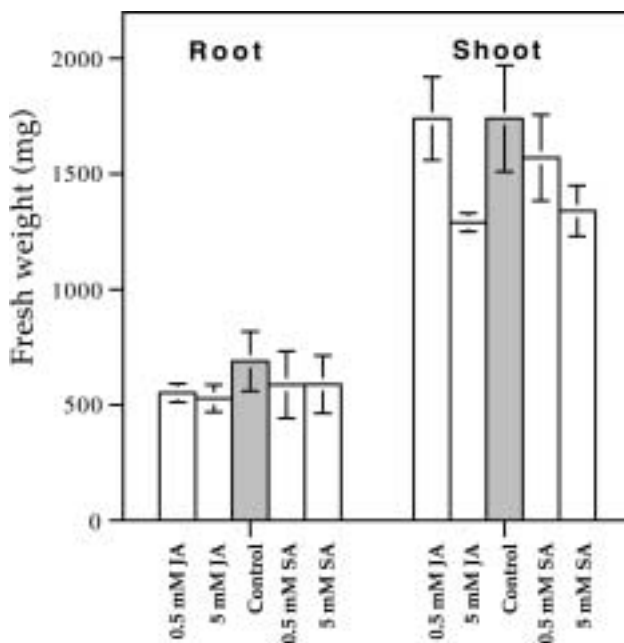


Figure 4. Root and shoot fresh weight of *Cucumis sativus* (cucumber) after foliar application of different concentrations of jasmonic or salicylic acid.

Table 2. GSL content in *T. canariense* and in different *T. majus* cultivars after inoculation (+ M) with *Glomus mosseae* (*Value from Table 1).

Species	Cultivar	Treatment	GSL	nmol g ⁻¹ DW	% infection
<i>T. majus</i>	Bot. Gard.	- M	benzyl 3-indolylmethyl	27441±307* 0*	0
		+ M	benzyl 3-indolylmethyl	63064±1637 248±23	42
<i>T. majus</i>	Empr. of India Dwarf	- M	benzyl 3-indolylmethyl	14314±669 222±40	0
		+ M	benzyl 3-indolylmethyl	42274±380 120±21	62
<i>T. majus</i>	Empr. of India Std.	- M	benzyl 3-indolylmethyl	19457±1124 389±54	0
		+ M	benzyl 3-indolylmethyl	51369±2006 248±23	70–80
<i>T. majus</i>	Alaska	- M	benzyl 3-indolylmethyl	13537±434 222±40	0
		+ M	benzyl 3-indolylmethyl	37993±2920 153±29	61
<i>T. majus</i>	Golden Gleam	- M	benzyl 3-indolylmethyl	16942±1153 243±31	0
		+ M	benzyl 3-indolylmethyl	50304±2611 311±27	73
<i>T. majus</i>	Scarlet Gleam	- M	benzyl 3-indolylmethyl	15585±525 190±11	0
		+ M	benzyl 3-indolylmethyl	67775±3141 596±346	54
<i>T. canariense</i>		- M	aliphatic	556±87	0
			p-OH-benzyl	457±11	
			benzyl	4861±359	
			p-MeO-benzyl	7674±98	
			3-indolylmethyl	0	
		+ M	aliphatic	trace	59
			p-OH-benzyl	4134±777	
			benzyl	14075±1495	
			p-MeO-benzyl	41385±1505	
			3-indolylmethyl	0	

treated plants, the mycorrhization was suppressed, thus excluding the implication of the observed GSL pattern changes in the reduced potential of JA treated plants to form the AM symbiosis. This is not surprising as the two accumulated GSLs, the aromatic GSL benzyl and the indole GSL 3-indolylmethyl, have been detected not only in GSL-containing AM non-host plants, but also in GSL-containing AM host plants (Vierheilig et al. 2000), thus making their involvement in the non-susceptibility of most GSL-containing plants to AMF unlikely.

In addition we could show that another GSL-containing plant in the family of the Tropaeolaceae, *T. canariense*, is a mycorrhizal host, as reported recently for *T. majus* (Vierheilig et al. 2000). Moreover, roots of all *T. majus* cultivars were highly colonized by the AMF *G. mosseae*. The increases of the GSL levels in mycorrhizal *T. majus* cultivars and in mycorrhizal *T. canariense* showed no effect on root colonization by the AMF. Vierheilig et al. (2000) recently suggested that rather

than the GSL levels, a specific qualitative GSL-pattern might be responsible for the AM-non-host status of most GSL-containing plants and proposed the aromatic GSL, 2-phenylethyl, occurring only in roots of AM non-host plants, but not in roots of GSL-containing AM host plants, as a good candidate to explain the non-mycorrhizal status of GSL-containing AM non-host plants. Unfortunately, with our study we can not provide further evidence for this hypothesis, as in none of the treatments 2-phenylethyl GSL was accumulated in the test plants.

A clear proof for a GSL-independent mechanism responsible for the observed suppressive effect of JA on mycorrhization was the observation in the GSL-free plant cucumber. In cucumber, as seen with *T. majus* and *C. papaya*, JA application to the leaves drastically reduced root colonization, whereas no effect on root colonization was observed after the SA treatment. The relatively low reduction of root colonization in *T. majus* might be explained by the waxy surface of its leaves, i.e. poorer absorption of applied compound. Even after rubbing the leaves moistening was probably not as homogeneous as on *C. papaya* and cucumber leaves. Plant growth (measured as root and shoot fresh weight) in some plants (*C. papaya* and cucumber) was affected by the higher concentration of the JA-solution (5 mmol/L), but not by lower concentrations (0.05 mmol/L or 0.5 mmol/L), thus excluding a simple plant growth suppression to be responsible for the observed suppression of AMF root colonization.

Despite the key role of the two signalling compounds jasmonic and salicylic acid in plant defense its role during the formation of the AM association has received little attention (recently reviewed by Ludwig-Müller 2000). Our results with JA seem in contrast to the work by Regvar et al. (1996) who foliar-treated garlic plants with JA and found a promotion of AM colonization. However, variation in methodology, and the nature of the test species, might explain the differing results. While we foliar-treated *T. majus*, *C. papaya* and cucumber plants every second day with a JA solution, Regvar and co-workers (1996) made one application per week on garlic plants. Moreover, we found a similar suppression of root colonization with different JA concentrations (0.05 mmol/L; 0.5 mmol/L or 5 mmol/L), while a promotion of mycorrhizal symbioses was observed with a lower concentration (5 µmol/L) (Regvar et al. 1996).

Recently a high accumulation of JA in mycorrhizal roots of cucumber has been reported and even in non-mycorrhizal roots of a split-root system of mycorrhizal cucumber plants increases of the JA levels could be detected (Vierheilig and Piché 2002). This increase was found during advanced stages of mycorrhization, so that it can be postulated that during later stages JA is needed to maintain the homeostasis between plants and AM fungi, whereas application of JA during early stages may contribute to the suppression of colonization. However, it should be noted that endogenous levels are not directly comparable with exogenously applied signaling substance. As JA is known to be involved in signal transduction in relation to defence gene induction (Gundlach et al.

1992), the systemic suppression of AMF root colonization in JA treated plants observed in this study and the local and systemic increase of JA levels in mycorrhizal roots reported before (Vierheilig and Piché 2002), could point towards a role of JA not only in local, but also in systemic suppression of root colonization by fungi in mycorrhizal plants.

After the application of SA to the roots a significant delay in mycorrhization has been reported recently (Blilou et al. 2000). As appressoria formation was not affected, a direct inhibitory effect of SA on the AMF was excluded. In our study we found a different picture. Although SA concentrations were comparable to those used by Blilou et al. (2000) in none of the tested plants any effect on root colonization could be detected. These differing results might be due to the different application site of the compound. Whereas Blilou et al. (2000) applied SA directly to the roots, thus looking at local effects of the compound, we applied it to the leaves, thus looking at possible systemic effects.

To summarize, our results show that changes of GSL levels in plants and the accumulation of new GSLs do not result in a changed host status of the plant to AMF, however, the role of specific GSLs in the expression of the non-host status of most GSL-containing plants, as suggested by Vierheilig et al. (2000) can not be excluded. Candidate substances are 2-phenylethyl-GSL or its derivative 2-hydroxy-2-phenylethyl-GSL. This hypothesis will be tested in our laboratory. JA and SA are both plant signal molecules important for induction and regulation of defense pathways. The adverse effect on root colonization by JA, but not by SA indicates an SA-independent plant defense mechanism responsible for the reduced susceptibility of JA-treated plants to AMF. It is clear that further studies are needed to elucidate the exact mechanism of AM suppression by JA under our experimental conditions.

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